United States Department of Agriculture
Grain Inspection, Packers and Stockyards Administration
Federal Grain Inspection Service

FGIS Issuance Change

CHANGE TO	☐ DIRECTIVE	☐ MANUAL	☒ HANDBOOK

CHANGE NO:	TO *(No.)*	TITLE:	DATE:
11		Aflatoxin Handbook	4-18-05

PURPOSE OF CHANGE: The Aflatoxin Handbook has been revised to include instructions for the Aflacard T20 test kit, to amend the Aflatest instructions pertaining to the biweekly check of the working standards, and to revise the extraction procedures for the Fast Aflatoxin SC test kit.

FILING INSTRUCTIONS

Remove	Dated	Insert	Dated
Table of Contents	1-31-05	Table of Contents	4-18-05
Page 1-3	1-31-05	Page 1-3	4-18-05
Page 1-4	No date	Page 1-4	4-18-05
Page 8-5	6-24-02	Page 8-5	4-18-05
Page 8-6	No date	Page 8-6	No date
Page 8-7	6-24-02	Page 8-7	4-18-05
Page 8-8	No date	Page 8-8	No date
Page 12-1	1-31-05	Page 12-1	1-31-05
Page 12-2	No date	Page 12-2	4-18-05
*********	******	Chapter 14	4-18-05

Retain this issuance sheet as an aid in verifying handbook contents.

/s/ David Orr

David Orr, Director
Field Management Division

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

TABLE OF CONTENTS

1.5 APPROVED TEST METHODS

FGIS has approved test kits for use at field testing locations. The Aflacard T20, AflaCup, and Agri-Screen and QuickTox test kits are approved for qualitative analysis of corn. The Aflatest, Fluoroquant, Veratox-AST, Myco✓, RIDASCREEN Fast Aflatoxin Total and RIDASCREEN Fast Aflatoxin SC test kits provide quantitative analysis but can be used for qualitative results. High Performance Liquid Chromatography (HPLC) testing is reserved for quantitative testing at the Technical Services Division (TSD) only.

FGIS APPROVED TEST METHODS			
Method and Test Kit	Approved for		
	Qualitative	Quantitative	Test Kit Range
Aflacard T20 – (R-Biopharm Rhone)	X		20 ppb
QuickTox - (Envirolix)	X		20 ppb
AflaCup - (International Diagnostics Inc.)	X		20 ppb
AgriScreen - (Neogen)	X		20 ppb
Veratox AST - (Neogen)	X	X	5 - 300 ppb
Fluoroquant - (Romer)	X	X	5 - 300 ppb
Aflatest – (Vicam)	X	X	5 - 300 ppb
Myco✓ - (Strategic Diagnostics Inc.)	X	X	5 - 80 ppb
RIDASCREEN Fast Aflatoxin Total - (r-Biopharm)	X	X	5 - 50 ppb
RIDASCREEN Fast Aflatoxin SC - (r-Biopharm)	X	X	5 - 100 ppb

NOTE: The test ranges are for performing an individual analysis with an undiluted sample extract. To obtain accurate results above the test kit range a supplemental analysis must be performed.

Listed in the table below are the test kits that are commonly used for official aflatoxin analysis. Use the table to determine the appropriate test kit(s) to use for testing the listed grain/commodity. For information concerning the testing of mixed grain, contact the Policies and Procedures Branch.

GRAIN/ COMMODITY	TEST METHOD									
	Aflacard T20	AflaCup	Aflatest	Agri-Screen	Fluoroquant	Myco✓	QuickTox	Ridascreen Fast Aflatoxin Total	Ridascreen Fast Aflatoxin SC	Veratox-AST
Corn	X	X	X	X	X	X	X	X	X	X
Sorghum			X		X	X		X	X	X
Wheat			X		X			X	X	X
Soybeans			X		X			X	X	X
Corn Screenings			(*)							(*)
Corn Meal			X		X	X		X	X	X
Corn Germ Meal			X					X	X	X
Corn Gluten Meal			X					X	X	X
Corn/Soy Blend			X		X	X		X	X	X
Corn Gluten Feed			X							
Flaking Corn Grits			X		(*)					(*)
Corn Flour									X	(*)
Corn Bran										(*)
Popcorn			X		X	X		X	X	X
Milled Rice			X		X			X		X
Rough Rice										(*)
Cracked Corn		(*)	(*)	(*)	(*)	(*)		X	X	(*)

NOTE: An X entered into a block denotes that the test kit has been evaluated and approved for the grain/commodity.

The symbol (*) entered into a block denotes that the test kit is under evaluation by TSD for the grain/commodity and is temporarily approved for official use.

(2) Biweekly check of working standards.

(a) Calibrate the fluorometer using the working set as described in "Calibration Procedures" (see section 8.3 b).

(b) Test the red and green vials from the working set and record the values.

(c) After calibrating the working set, remove the reference set from storage and test the 3 vials as described in section 8.3, b. The difference in readings of the two sets should not exceed the following limits:

Red	Yellow	Green
± 10 ppb	± 5 ppb	± 2 ppb

If the difference between the working and reference sets exceeds the tolerances, discard the working set. Begin using the old reference set as the working set, and use the new set as the reference set. Keep a permanent record of all calibration verification data.

8.4 SOLUTION TESTING

The distilled/deionized water, dilute developer solution, and HPLC grade methanol must be tested for background fluorescence before use. After calibrating the fluorometer perform the following:

a. Methanol.

Place 2.0 ml of HPLC grade methanol into a clean cuvette. Place the cuvette in the calibrated fluorometer. The displayed reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, replace the methanol.

b. Water.

Dispense 2.0 ml of deionized/distilled water into a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, take action to assure a pure water supply.

c. Developer Solution.

Combine 1.0 ml of dilute developer solution and 1.0 ml of HPLC grade methanol in a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0.

If the reading is positive and greater than 1.0, check each reagent separately to determine which reagent is causing the problem and replace it.

8.5 TEST PROCEDURES

a. Procedures for Testing Corn, Corn Meal, Corn/Soy Blend, Flaking Corn Grits, Milled Rice, Popcorn, Sorghum, and Soybeans.

Note: All aflatoxin tests for rice are performed on a milled rice basis. Consequently, rough rice or brown rice require milling before analysis. Mill rough rice or brown rice according to the procedures in the Rice Handbook.

(1) Extraction.

(a) Place 50 g of ground sample into blender jar.

(b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.

(c) Add 100 ml of the 80/20 methanol/water extraction solution.

(d) Cover jar and blend at high speed for 1 minute.

(e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.

(f) Collect the filtrate in a clean beaker labeled with the sample identification.

(g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.

(h) Pipette 5 ml of filtered extract into a clean beaker.

(i) Add 10 ml of distilled/deionized water and mix thoroughly.

(j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.

(k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

(a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.

(b) Using an Eppendorf pipette, or equivalent, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.

(c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

(d) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.

(e) Repeat the water wash in step (d) above.

(f) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicam Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.

(g) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.

(h) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.

(i) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).

(j) **Immediately** place the cuvette in a calibrated fluorometer.

(3) Reading, Recording, and Certifying Test Results.

(a) Record the digital readout (Series III and IV) or corresponding bar graph value (MF-2000) as total ppb.

(b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.

(c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.

(d) Refer to the Certification section of the handbook for more detailed certification procedures.

12.1 GENERAL INFORMATION

The RIDASCREEN® FAST Aflatoxin SC test is a competitive enzyme immunoassay for the quantitative analysis of aflatoxin in select grains and commodities. **The test kit is limited to providing aflatoxin measurements between 5 – 100 ppb.**

12.2 PREPARATION OF SOLUTIONS

a. Extraction Solution.

The extraction solvent used in the RIDASCREEN® FAST Aflatoxin SC test is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS grade or better) and 30 percent water.

(1) Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.

(2) Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.

(3) Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.

(4) Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

b. Wash Solution.

(1) Dissolve the contents of the packet containing the buffer salt in 1 liter of distilled water.

(2) Swirl to mix.

(3) Store this solution in a refrigerator until needed. The solution expires 4 weeks after preparation.

12.3 EXTRACTION PROCEDURES

a. Transfer 50 grams of ground sample into an extraction mixing jar.

b. Add 250 ml of the (70/30) methanol/water extraction solvent.

c. Cover the extraction jar and blend on high speed for 2 minutes.

d. Filter approximately 1.5 ml of the extract through a filtering syringe or equivalent.

e. Dilute 1 ml of the filtrate with 1 ml of distilled or deionized water.

f. Proceed to test procedures.

12.4 TEST PROCEDURES

a. Sample Analysis.

(1) Allow reagents, microwells, and sample extracts to reach room temperature prior to running the test.

(2) Insert a sufficient number of wells into the microwell holder for all standards and samples to be tested. (For example: to test 7 samples use 8 wells - 1 for the standard and 7 for the test samples).

Test Strip

Well #	1	2	3	4	5	6	7	8
Sample	C 0	S1	S2	S3	S4	S5	S6	S7

Where C 0 is the zero control, S1 is sample 1, S2 is sample 2, S3 is sample 3, etc.

NOTE: Do not run more than 3 strips (23 samples) per set of control standards.

(3) Using a new pipette tip for each standard and sample, pipet 50 µl of standard and prepared sample to separate wells.

(4) Add 50 µl of enzyme conjugate (red capped bottle) into each well.

(5) Add 50 µl of anti-aflatoxin antibody (black capped bottle) into each well.

CHAPTER 14

AFLACARD T20 TEST KIT

14.1 GENERAL INFORMATION

The AFLACARD T20 test kit is a qualitative enzyme immunoassay procedure for the detection of total aflatoxins. The test provides qualitative (less than or equal to a specified threshold) results.

14.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the AFLACARD T20 test method is a methanol/water (distilled or deionized) mixture consisting of 80 percent methanol (Reagent grade or better) and 20 percent water.

a. Using a graduated cylinder, measure 800 ml of methanol and place it into a clean carboy with spigot.

b. Add 200 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.

c. Label the container stating the mixture (80 percent methanol and 20 percent water), date of preparation, and initials of technician who prepared the solution.

d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 8 parts methanol to 2 parts of deionized or distilled water.

14.3 PREPARATION OF TESTING MATERIALS

a. Conjugate.

(1) Add 2 ml of conjugate diluent buffer (pink label) to the freeze-dried conjugate (amber vial).

(2) Replace the rubber cap and mix gently by inversion.

(3) Transfer all of the conjugate into the empty conjugate dropper bottle (red label) and write the preparation date on the label.

(4) **Leave the conjugate at room temperature for at least 30 minutes before use.**

NOTE: The ready to use conjugate is stable at 36° - 46° F

b. Other Kit Components.

(1) Remove the AFLACARD T20 kit from the refrigerator and leave the test kit components; substrate (blue label), wash buffer (green label), substrate (blue label), stop solution (yellow label), and test card at room temperature for at least 30 minutes before using the test.

Each card has two ports and therefore can perform two tests. The second port should be used within 8 hours of the first port. Each port has a sample area and control area. Please ensure the airholes on the card are on the right hand side and are not blocked or covered during the assay.

(2) Check that the two ports on the card to be used each exhibit two light blue spots.

NOTE: Each unused card has two light blue spots on each port which will disappear during the course of the assay.

14.4 EXTRACTION PROCEDURES

a. Transfer 50 grams of ground sample into an extraction mixing jar.

b. Add 100 ml of the (80/20) methanol/water extraction solvent.

c. Cover the extraction jar and blend on high speed for 1 minute.

d. Remove the cover and funnel a minimum of 10 ml of the extract through a Whatman No.4 filter paper into a sample jar labeled with the sample identification.

e. After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container.

14.5 TEST PROCEDURES

a. Sample Preparation.

(1) Remove the lid from the sample diluent tube and add 200 µl of filtrate.

(2) Cap and invert sample diluent tube. The sample is now ready to be applied to the card.

b. Sample Analysis.

(1) Apply 250 µl of diluted filtrate to sample port and incubate for a minimum of 1 minute. Always ensure that the liquid has passed completely through the membrane before proceeding to the next step.

(2) Using the conjugate dropper bottle (red label), apply 3 drops of conjugate to the test port and incubate for 1 minute.

(3) Using the wash buffer dropper bottle (green label), apply 3 drops of wash buffer to the test port and incubate for 1 minute.

(4) Dry around the port with a tissue.

(5) Using the substrate dropper bottle (blue label), apply 3 drops of substrate to the test port and incubate for 2 minutes.

(6) Using the stop solution dropper bottle (yellow label), apply 3 drops of stop solution to the test port. Allow the solution to pass completely through the membrane.

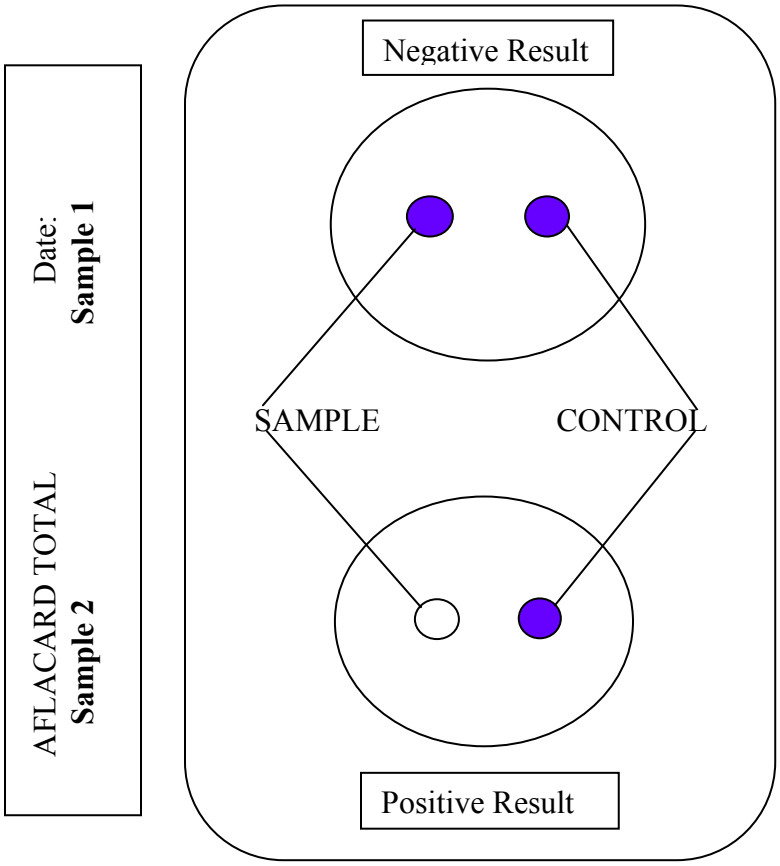
c. Reading Test Results.

(1) The control spot must develop a **clearly visible purple color** in order to have a valid test result. The color in the sample and the control spot does not need to be of the same intensity.

(2) The sample should be considered to be negative when the sample and control spot both have clearly visible color development.

(3) The sample should be considered to be positive (more than 20 ppb) when there is no detectable color on the sample spot.

INTERPRETATION OF RESULTS



14.6 REPORTING AND CERTIFYING RESULTS

a. Report results on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.

b. Certify results as being equal to or less than a threshold.

c. Refer to the Certification section of the handbook for more detailed certification procedures.

14.7 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

14.8 WASTE DISPOSAL

a. Negative Results (≤ 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

14.9 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits:

(1) 10 Aflacard Total Cards.

(2) 20 tubes containing 3.8 ml Sample Diluent Buffer.

(3) 2 Freeze-dried Conjugate vials (red label).

(4) 1 Conjugate Diluent Buffer vial (pink label).

(5) 2 Conjugate Dropper Bottles (empty, red label).

(6) 1 Wash Buffer dropper bottle (green label).

(7) 1 Substrate dropper bottle (blue label).

(8) 1 Stop Solution dropper bottle (yellow label).

b. Materials Required but not Provided:

(1) Sample grinder.

(2) Balance.

(3) Methanol - ACS grade.

(4) Distilled or deionized water.

(5) Blender with mixing jars.

(6) Timer.

(7) Whatman No.4 Filter Paper.

(8) Tissue paper.

(9) Sample collection container.

14.10 STORAGE CONDITIONS

a. Storage Conditions.

Test kits should be refrigerated between 36° - 46° F. **Do not freeze.**

b. Precautions.

(1) Do not use kit components beyond the expiration date.

(2) Do not use reagents from one batch number in conjunction with reagents from a different batch number, and do not substitute reagents from other manufacturers.

(3) Kits should be brought to room temperature (68° - 82° F) prior to use. This will take approximately 30 minutes.